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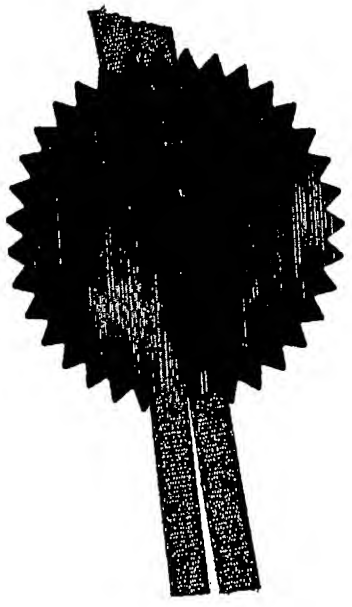

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1. Your reference GBP88353

2. Patent application number (The Patent Office will fill in this part) 0317656.7

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Oxitec Limited,
2nd Floor
Park Gate
25 Milton Park
Oxford OX14 4SH
United Kingdom

Patents ADP number (if you know it) 8571051001

If the applicant is a corporate body, give the country/state of its incorporation: United Kingdom

4. Title of the invention: Pest Control

5. Name of your agent (if you have one)
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Marks & Clerk
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Patents ADP number (if you know it) 18001

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Country	Priority application No (if you know it)	Date of filing (day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing (day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:
a) any applicant named in part 3 is not an inventor, or
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11. I/We request the grant of a patent on the basis of this application.

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Patent Chemical Formalities
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PEST CONTROL

The present invention relates to insect expression systems comprising a promoter.

The genetic manipulation by recombinant DNA methods of insect species other than *Drosophila melanogaster* is in its infancy (Alphey, 2002; Alphey and Andreasen, 2002; Alphey *et al.*, 2002; Berghammer *et al.*, 1999; Catteruccia *et al.*, 2000; Coates *et al.*, 1998; Jasinskiene *et al.*, 1998; McCombs and Saul, 1995; Peloquin *et al.*, 2000), and very few transgenic lines of non-*Drosophila* insects have been made, using heterologous promoters.

Insect transformation is a low-efficiency system requiring the identification of rare transformants in a background of larger numbers of non-transformed individuals. It is, therefore, important that the transformants have an easily scored marker. The current favourites are the fluorescent proteins, GFP, DsRed and their mutant derivatives. These require transcriptional control elements, including a promoter, for their function. The best known of these are from the *Drosophila* Actin5C (Act5C) and ubi-p63E (Pub) genes. A silk moth homologue of Act5C, BmA3, has also been used, as well as a couple of tissue-specific promoters (3xP3, a synthetic eye-specific promoter, and Act88F, specific to the indirect flight muscles).

However, none of these promoters is entirely satisfactory. Act5C has been used to transform various mosquitoes, as well as *Drosophila*, but its expression pattern in mosquitoes is far from ubiquitous. Efforts to use it as part of a transformation marker in medfly (*Ceratitis capitata*) have failed, where equivalent experiments with Pub have achieved good success. Pub has similar limitations: the expression pattern seen in medfly transformants is highly variable, suggesting that the expression pattern is at least highly sensitive to position effect. In addition, none of these promoters can be regulated in the sense of being turned on and off as desired.

Fussenegger *et al.*, Cytotechnology 1998 illustrate positive feedback driving multi-cistronic transcripts, using a selection marker, in one instance. Experiments were restricted to mammalian systems. pTRIDENT is described as a tricistronic artificial mammalian operon. Expression or transient expression of cell cycle arresting genes is described for "metabolic engineering", *i.e.* regulating expression of desirable proteins, and it is mentioned that a transcriptional "squelching" effect by the VP16 transactivator domain may be lethal for the host

cell, even at moderate expression levels (Gill and Ptashne, 1988). The benefits of autoregulatory mono- or poly-cistronic systems are discussed, including one-step, auto-regulated and auto-selective multicistronic mammalian expression systems which included the tTA in a multicistronic, pTRIDENT-based or quattrocistronic configuration (pQuattro-tTA; Fussenegger *et al.*, 1997b; Figure 2). Since the tTA gene is encoded on the multicistronic expression unit itself, little or no tTA is expressed under repressive conditions. This positive feedback regulation system showed no signs of squelching. Experiments with a monocistronic positive feedback configuration in transgenic animals also showed no detrimental effects (Shocket *et al.*, 1995).

Very few promoters or other control elements have been characterised, and there remains a pressing need for such elements. It is an aim of the present invention to provide a universal promoter active in all or most cells of a wide range of insects. It is a further aim to regulate the activity of such promoters, especially in a life stage- and/or sex-specific manner. It is also an aim to selectively reduce or eliminate the promoter activity in particular cells or tissues. The present invention provides such systems.

Surprisingly, it has now been found that it is possible to employ a positive feedback mechanism both to enhance the effect of an insect promoter as well as to control its expression.

Thus, in a first aspect, the present invention provides an insect gene expression system, comprising at least one gene to be expressed and at least one promoter therefor, wherein a product of a gene to be expressed serves as a positive transcriptional control factor for the at least one promoter, and whereby the product, or the expression of the product, is controllable.

As used herein, the term "gene" refers to any DNA sequence that may transcribed or translated into a product having activity *in vivo*.

The product capable of positive transcriptional control may act in any suitable manner. In particular, the product may bind to an enhancer located in proximity to the promoter or promoters, thereby serving to enhance polymerase binding at the promoter, for example. Other mechanisms may be employed, such as repressor countering mechanisms, such as the blocking of an inhibitor of transcription or translation. Transcription inhibitors may be blocked, for

example, by the use of hairpin RNA's or ribozymes to block translation of the mRNA encoding the inhibitor, for example, or the product may bind the inhibitor directly, thereby preventing inhibition of transcription or translation.

More preferably, the mechanism is a positive feedback mechanism, wherein the product, which may either be RNA or the translation product thereof, acts at a transcription enhancer site, normally by binding the site, thereby enhancing promoter activity. Enhancement of the promoter activity then serves to increase transcription of the gene for the product which, in turn, further serves to either lift inhibition or enhance promotion, thereby leading to a positive feedback loop.

Control of the product may be by any suitable means, and may be effective at any level. In particular, it is preferred that the control be effective either to block transcription of the control factor gene or to block translation of the RNA product thereof, or to prevent or inhibit the action of the translation product of the gene.

For example, the gene product of tTA (tetracycline-repressible transcription activator) acts at the tetO operator sequence. Upstream of a promoter, in either orientation, tetO is capable of enhancing levels of transcription from a promoter in close proximity thereto, when bound by the product of the tTA gene. If the tTA gene is part of the cassette comprising the tetO operator together with the promoter, then positive feedback occurs when the tTA gene product is expressed.

Control of this system is readily achieved by exposure to tetracycline, which binds to the gene product and prevents transactivation at tetO.

The tTA system also has the advantage of providing stage-specific toxicity. In particular, "squelching" is observed in the development phases of insect life, the precise phase being dependent on the insect. Some insects may reach pupation before the larva dies, while others die early on. However, in general, adult insects appear to be immune to the squelching effect of tTA, so that it is possible to raise insects comprising a tTA positive feedback system in the presence of tetracycline and then to release the adult insects into the wild. These insects are at little or no competitive disadvantage to the wild type, and will breed with the wild type insects, but larvae carrying the tTA positive feedback cassette will die before reaching maturity.

Thus, the present invention is useful in combination with a dominant lethal gene, allowing selective expression of the dominant lethal gene, or stage specific expression, as desired, of the lethal gene or the lethal phenotype. It will be appreciated that the dominant lethal gene does not need to be an integral part of the positive feedback mechanism, but may be part of a bicistronic cassette, for example. Use of the present invention in association with RIDL (Release of Insects carrying the Dominant Lethal) is particularly preferred.

Control of the feedback mechanism, in this case, is simply effected by the presence or absence of tetracycline, or by modulating tetracycline concentration, when the tTA gene product is used. In the case of GAL4, this may be controlled by temperature, for example, thereby suppressing the effective gene, preferably a dominant lethal gene, until release of the insect.

Other mechanisms may also be employed, such as ribozymes or antisense or partially self-complementary RNA molecules, such as hairpin RNA, to inhibit or prevent expression of an activating peptide, or blocking agents that prevent binding of the activator to the enhancer site.

Such blocking agents may be expressed by the insect itself under selective conditions, or may be administered as part of the culture medium, for example.

Where the blocking, or controlling agents are produced by the insect, then it is preferred that their expression be selective, such as being sex specific. Administration of the blocking agent in the culture medium, for example, will enable suppression of the positive feedback cassette under all circumstances until release of the insect, after which stage- or sex- specific selection will occur.

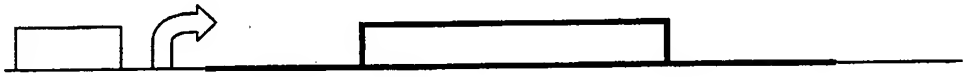
More preferably, the cassette comprising the positive feedback mechanism is associated with stage- or sex- specificity. For example, sex specific splicing is observed with the *transformer* and *doublesex* mechanisms, and can be employed to limit expression of the feedback system to a particular sex, either by employing sex specific splicing to delete all or part of the effector gene, or to incorporate a frameshift or stop codon, for example.

Although it is possible to provide the effector gene in a separate location and even on a separate chromosome, it is generally preferable to link the effector gene with the feedback gene. This may be achieved either by placing the two genes in tandem, including the possibility of providing the two as a fusion product, or for example by providing each gene with its own promoter in opposite orientations but in juxtaposition to the enhancer site.

It is preferred to include a marker with the systems of the invention, such as DsRed, green fluorescent protein, and variants thereon, as transformation success rates in insects are extremely low, so that it is useful to be able to select in some way.

The promoter may be a large or complex promoter, but these often suffer the disadvantage of being poorly or patchily utilised when introduced into non-host insects. Accordingly, it is preferred to employ minimal promoters, such as the Hsp70 promoter which, while having a naturally somewhat low level of activity, can be substantially enhanced by a positive feedback scenario, such as by the use of tTA.

Thus, in a preferred aspect, the present invention provides positive feedback constructs of the general form shown below:



$tetO_7$ minimal promoter

tTA coding region

other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA

The tetracycline-repressible transcription activator (tTA) protein, when expressed, binds to the tetO operator sequence and drives expression from a nearby minimal promoter. In the configuration shown above, this then drives expression of tTA, which then binds to tetO, and so on, creating a positive feedback system. This system is inhibited by tetracycline, which binds to tTA and prevents it binding tetO.

Regulated expression may be achieved by operably linking the promoter to a controllable transcription factor, such as tTA (tetracycline-repressible or tetracycline-inducible), GAL4

(which is somewhat cold-sensitive, and can be further controlled by use of GAL80 or mutants thereof), or the streptogrammin regulated expression system. It will be appreciated that other binding sites for the appropriate transcription factor will depend on the transcription factor concerned, such as UAS_{GAL4} for GAL4, for example.

Preferred systems of the present invention have high levels of induced expression, preferably available at several induced levels, with a low basal level of expression of the regulated gene but also of any other component, and preferably across a range of species.

Different constructs of the invention have varying activity, according to the components of the constructs. For example, in *Drosophila*:
WTP296AiK-tTA gives a low level of induced (non-repressed) expression
JY2004-tTA gives strong expression when not repressed, approximately equivalent to Act5C-tTA
LA513 is lethal when not repressed.
The first two appear to give constitutive expression, as judged by use of a reporter gene (tRE-EGFP), this is difficult to assess for the lethal LA513, although at 10μg/ml tet, just sufficient for good survival, LA513 in *Drosophila* drives expression of a $tetO_7$-EGFP reporter gene in both the male and female germline in adults, as well as in somatic cells. This distinguishes it from Act5C, commonly used as a "ubiquitous, constitutive" promoter, which does not, in fact, express in these cells.

The properties of these constructs are shown in Table 1, below.

Table 1

	Max expression	Minimal promoter	Intron	Optimised coding region?	3'UTR and polyA
WTP296AiK-tTA	Low	P	PP1α96A	No	*fs(1)K10*
JY2004-tTA	High	CMV	Rabbit β-globin	No	Rabbit β-globin
LA513	V. high (lethal)	Hsp70	Adh	Yes	*fs(1)K10*

Accordingly, it will be appreciated that the induced or non-repressed expression level can be modified in a useful and predictable way by adjusting the sequence of the positive feedback system. Toxicity and/or activity of the tTA protein can be modified independently of the transcriptional and translational control signals by several approaches, *e.g.* use of a nuclear localisation signal, modification of the activation domain, *etc.* (*c.f.* Fussenegger, 2001).

The lethality of LA513 is useful, for the reasons given above, and more particularly because:

a) It provides a compact, highly effective repressible lethal gene system;

b) As it uses only simple control elements from *Drosophila* (hsp70 minimal promoter, a small intron and the K10 terminator), it functions across a wide phylogenetic range, as also demonstrated by JY2004-tTA, which has no insect components in its positive feedback cassette;

c) It has very little, if any, deleterious effect on adults, even in the absence of tetracycline. This is a highly desirable and surprising property for field use, for example in a RIDL-based control programme, as the released adults must be competitive and long-lived for full efficacy of the programme. It will be appreciated that the effect of the system of the invention could be further modified by the incorporation of an adult-effective lethal, for example in the "positive feedback – bi-directional expression" configuration described herein; and

d) By its nature, "cross-talk" between various elements is minimised. This is because: (i) the core of the construct is only a single composite element, rather than the normal two in bipartite expression systems; (ii) the principal enhancer of the autoregulatory component, the tTA binding sites, is substantially active only in the absence of tetracycline and (iii) modest expression of tTA under the influence of a nearby enhancer, whether in another part of the construct or in nearby chromatin, is unlikely to be significantly deleterious.

Without being bound by theory, the mechanism by which LA513 kills embryos and early larvae but not adults appears to be an inherent property of its toxicity. tTA toxicity is believed to derive from "transcriptional squelching", in which high level expression of the transcriptional activator domain (in the case of tTA this is VP16 or a fragment thereof) binds elements of the transcriptional machinery and titrates them, leading to a general effect on transcription, although it may also act to saturate the ubiquitin degradation pathway. Transcriptional squelching is the effect which is thought to lead to deleterious effects in mammalian cell lines expressing tTA at high levels; in the optimised expression context of LA513 positive feedback drives tTA

expression to lethal levels. However, developing stages may be more sensitive to disruption of transcription than adults: they have to express genes in a highly coordinated fashion to allow proper development, while adults may be more tolerant of disruption.

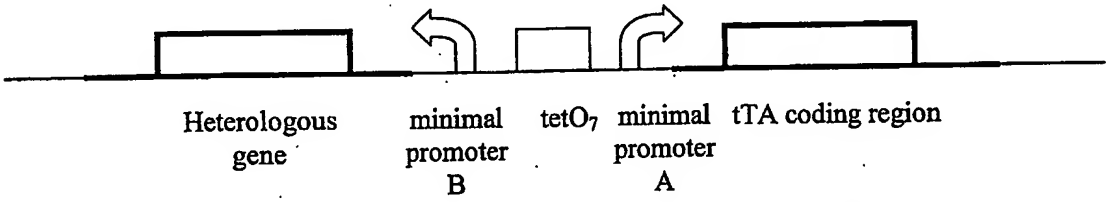
The development of LA513 heterozygotes on media with an intermediate level of tet (3 or 10 μg/ml), just sufficient for survival, showed a significant delay, relative to their wild type siblings. Parallel experiments using higher concentrations of tet, *e.g.* 100 μg/ml, did not show any developmental delay, thereby suggesting that sub-lethal expression of tTA can adversely affect the normal development of the insects.

It is preferred that a positive feedback system show a higher on:off ratio and switch from on to off over a narrower concentration range than a conventional system, thereby allowing the use of a wider range of effector molecules. Lower-toxicity (lower specific activity) effector molecules can be used, as they can be expressed at a high level under active conditions without leading to problems of toxicity at basal levels. Conversely, more toxic (higher specific activity) ones can be used as the necessary low basal level does not preclude high levels of expression when de-repressed or induced. Since basal level of expression is only partly determined by the level of tTA, this advantage is particularly clear in the case of lower-toxicity molecules. tTA is a preferred example of a low specific activity effector molecule that can be used as a lethal in the positive feedback context of LA513, for example. The advantage of switching from on to off over a narrow concentration range is that a modest concentration of repressor can be used without risk of residual (not fully repressed) expression leading to adverse effects and potentially selecting for resistance. Conversely, for an inducible system, modest concentrations of the activator can give full expression.

Activated or de-repressed drivers are useful for expressing effector molecules. Examples of effector molecules include functional RNA's, such as hairpin RNA's, ribozymes *etc.*, and one or more encoded proteins. It will be appreciated that, for different applications, different levels of expression are appropriate. Since the sequence-specific transcription factors used to drive the positive feedback system can also be used to express other genes in a bipartite expression system, this may be achieved by making two separate constructs, one with the driver (normally a promoter-transcription factor construct, here the positive feedback construct), the other with the gene or molecule of interest under the control of a composite promoter (binding site + minimal

promoter) responsive to the transcription factor (Bello *et al.*, 1998; Brand *et al.*, 1994). This is also appropriate for these positive feedback drivers. Alternatively, the two elements may be combined on the same construct. This embodiment has significant advantages for most field applications, as it very substantially reduces the risk that the two functional elements can be separated by recombination. Further, the complete expression system can be introduced with only a single transformation event, as well as meaning that insects homozygous for the system are homozygous at only one locus rather than two, which makes them easier to construct by breeding, and tends to reduce the fitness cost due to insertional mutagenesis.

It is also possible to condense such an expression system into a more compact form, such as illustrated below:



This exploits the bi-directional nature of enhancers, in this case the tetO binding site in the presence of tTA. This arrangement further allows, or facilitates, the use of insulator elements to reduce the effect of enhancers or suppressors in the adjacent chromatin: in this arrangement the entire expression cassette can be flanked by insulators. This arrangement also removes the need to duplicate the transcription factor binding sites within the construct. Such duplication is preferably avoided, as it can lead to instability through homologous recombination. For similar reasons, it is generally preferred that non-identical insulators, such as scs and scs' are used, rather than using the same one twice.

It is further possible to condense the system to provide a single transcript, either bicistronic or expressing a single polypeptide, which may potentially be further processed into more than one protein. Each of these approaches (bi-directional expression, bicistronic expression, fusion protein with transactivator) tends to reduce the size of the construct, which in turn will tend to increase the transformation frequency and reduce the mutagenic target.

As an example of the utility of such a system, a general transformation marker might be constructed by using a transactivator system known to function over a wide phylogenetic range, for example those based on tetR, GAL4, lexA or AcNPV ie-1. Such a transactivator,

functionally linked to a coding region for a fluorescent protein by any of the above methods (bi-directional expression, bicistronic expression, fusion protein with transactivator), would provide a genetic marker expressed in a wide range of tissues and developmental stages across a broad phylogenetic range. Such a marker would be useful not only for detecting transgenics in transformation and other lab experiments, but also for distinguishing, for example, transgenic flies from wild type flies in the field, or those caught in the field.

Another example is expression of a transposase. Integrated into the chromosomes, this would be a "jump-starter" construct, for example *piggyBac* transposase integrated into an insect chromosome using *mariner/mos1*. Such constructs are useful to remobilise *piggyBac* elements. A widely-applicable jump-starter should be expressed at a significant level across a wide phylogenetic range. The expression system of this invention provides this. Furthermore, such a construct (*piggyBac* transposase under the control of a positive feedback system of one of the above structures) would also be useful in insect transformation *via* transient expression (co-expression of a "helper" plasmid, the most widely-used method for insect transformation), and again would be useful and functional across a wide phylogenetic range.

It is advantageous to regulate the action of an expression system at stage-, sex- or other levels, in addition to being able to regulate the expression level by changing environmental conditions. Suitable examples are as follows:

1. Expression of a repressor protein.

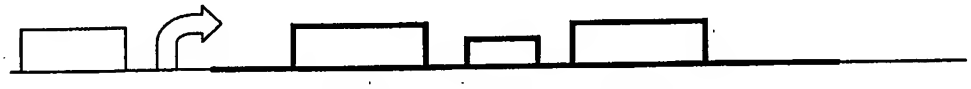
Repressor proteins are known or can be constructed for the main expression systems, *e.g.* GAL80 or its mutant derivatives for the GAL4 system, tetR fused to inhibitory proteins for the tet system, *etc.* Another alternative is gene silencing of the transcription factor using a hairpin RNA directed against part of the expression cassette. Basal expression from the positive feedback system is rather low, therefore it can readily be suppressed by expression of such an inhibitor.

Expression of a suitable inhibitor under suitable control will tend to inhibit expression from the positive feedback expression cassette where the inhibitor is expressed. Female-specific expression, for example, can therefore be achieved by expressing an inhibitor in males.

2. Integrating specificity into the positive feedback system

Specificity can be integrated into the positive feedback system by using components that are themselves specific. For example, the hsp70 minimal promoter + SV40 intron and polyA signal combination of pUAST is known not to be expressed in the female germline of *Drosophila*, while the P minimal promoter + P intron + *fs(1)K10* polyA signal of pUASp is so expressed (Rorth, 1998). Positive feedback expression systems can, therefore, be constructed which specifically do or do not express in this tissue, depending on the use of appropriate regulatory elements.

In another embodiment, sex-specificity can be integrated into the system by use of sex-specific splicing. The sex-specific splicing of *doublesex* and its homologues is a conserved regulatory mechanism and, therefore, available for use in this way across a wide phylogenetic range. Sex-specific splicing of *transformer* and its homologues is another alternative. The use of sex-specific splicing to integrate specificity into a positive feedback expression system can be achieved in several ways, as shown diagrammatically below. Appropriate extensions to and variations of the arrangements shown diagrammatically will be apparent to those skilled in the art.

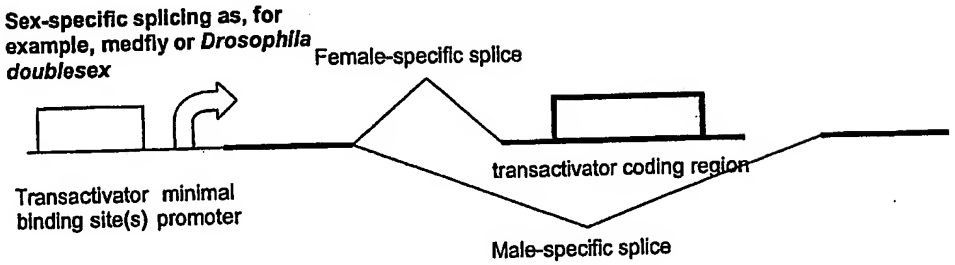


One ORF encodes transactivator

other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA

Fusion protein coding region

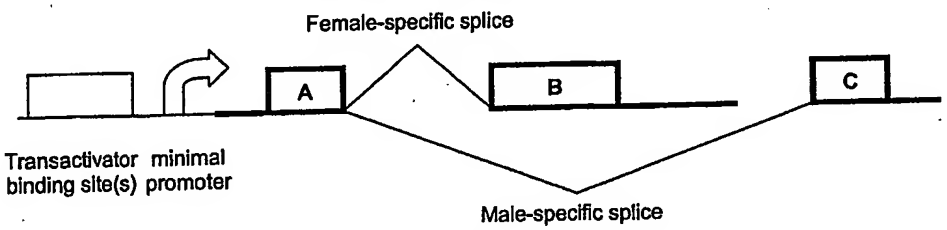
other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA



Other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA

Transactivator coding region:
A + C = transactivator
B = contains stop codon
or
A = DNA binding domain
B = Repression domain
C = Activation domain
Other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA

In another configuration, a specific splice site can be inserted into the transactivator coding region so that two (or more) alternative proteins are produced in different conditions, *e.g.* in different cell types or in different sexes. This can be arranged so that a transcriptional activator is produced in one cell type but a transcriptional repressor is produced in another cell type. This arrangement has the advantage that it is relatively robust to inefficient (imperfect) splicing – production of a relatively low proportion of transcriptional activator in the inappropriate cell type, *e.g.* in male cells, will be less likely to produce the positive feedback amplification as these cells are also producing a larger amount of repressor. Discrimination in output (ratio of levels of transcriptional activator in the two cell types, or ratio of expression of a reporter or other RNA or protein functionally linked to the expression of the transcriptional activator) between the two cell types is thereby enhanced.



Transactivator coding region:
A = DNA binding domain
B = Activation domain
C = Repression domain
Other control signals not marked, *e.g.* 5'UTR, 3'UTR, intron(s), polyA

It will be readily apparent to those skilled in the art that any of these specific transactivator arrangements can readily be combined with any of the arrangements disclosed herein for expression of an additional protein or RNA, *e.g.* bi-directional expression, bi- or multi-cistronic expression, expression of a fusion protein, or combined with one or more separate expression cassettes dependent on, or partly dependent on, expression of the transactivator, either combined on the same construct or elsewhere in the genome or cell.

3. Using a specific effector molecule

Specificity in phenotypic consequence can also be introduced by use of a specific effector molecule. Where a molecule, *e.g.* RNA or protein, expressed under the control of any of the expression systems described herein, has a specific effect only in particular cells, tissues, or sex, *etc*, then phenotypic specificity can be obtained with broader or less specific expression of the transactivator. For example, in the context of a RIDL-type mass-release insect population control programme, using the system to express a molecule only toxic, or preferentially toxic, to pre-adult stages, results in adults which are fully, or reasonably competitive, relative to wild type. This is desirable as the effectiveness of the programme depends on the competitiveness and longevity of the adult forms, when released into the wild. Since their internal repressor (*e.g.* tetracycline) concentration is likely to decline in the wild, it would be advantageous to ensure that induction (de-repression) of the expression system, as and when it occurs in adults, has a minimal negative effect on them.

As another example, sex separation, or sex-specific effects, can be achieved by expression in both males and females of a molecule with differential effects in males and females. For example, expression of the Transformer protein in male *Drosophila* will tend to transform them into females, but have no effect on females. Similarly, expression of Male specific lethal-2 (Msl-2) protein in *Drosophila* will tend to kill females, but not males (Gebauer *et al.*, 1998; Kelley *et al.*, 1995; Matsuo *et al.*, 1997; Thomas *et al.*, 2000). Conversely, expression of a partially self-complementary RNA molecule with substantial homology in its self-complementary or double-strand-forming region to ("hairpin RNA against") *transformer* will tend to transform genetic females into phenotypic males, while not affecting genetic males, and expression of hairpin RNA against *msl-2* will tend to be lethal to males but not to females. Expression of hairpin RNA against the male- or female-specific exons of *doublesex* will tend to affect those sexes only, and simultaneous expression of RNA encoding the other form of

doublesex (*i.e.* Dsx^M in females or Dsx^F in males) will tend to modify or enhance this effect. This simultaneous expression of a protein and a hairpin RNA molecule can readily be accomplished by combining the bicistronic or fusion protein approach described above with expression of a hairpin RNA using the bi-directional expression system also described above. Sex-, stage- or other specificity can be further added to such a system by incorporation of appropriate specific splicing or other transcriptional, translational or other post-translational control signals to either part of the system as will be apparent to the person skilled in the art.

Multi-functional hairpin RNA molecules may be constructed and are envisaged. For example, RNAi against *transformer* in the Mediterranean fruit fly *Ceratitis capitata* Wiedmann (medfly) will tend to transform genetic females into fertile males. For an area-wide population control program based on mass-release of such insects, it is preferable to sterilise the released flies. This can be accomplished by using a composite RNA molecule that simultaneously disrupts expression of both *transformer* and a gene required for spermatogenesis or embryonic or larval viability. Many such genes are known in *Drosophila* with homologues in mosquitoes or other animals. With medfly, a suitable homologue can readily be isolated, using techniques known to those skilled in the art. We prefer the use of a gene which allows the production of seminal fluid, and preferably also of sperm, to reduce the tendency of the female to re-mate after insemination by the affected male. We particularly prefer to direct this second part of the hairpin RNAi molecule against a paternal effect lethal, so that no viable progeny can be produced, or against a zygotically expressed gene required for embryonic or larval viability or development, so that progeny inheriting the construct will be affected. Other configurations are envisioned and will be readily apparent to those skilled in the art: for example expression of a female-specific lethal protein by bicistronic expression and a hairpin RNA leading to paternal-effect lethality by bi-directional expression. In common with the composite hairpin RNA against a suitable sex-determination gene and a paternal effect lethal, this allows the generation of a single-sex (male-only) population of insects, all of whose progeny die through the action of the paternal-effect lethal, irrespective of whether their progeny or mates feed on tetracycline. Thus, the present invention provides a controlled promoter, as defined, wherein the promoter is operably linked with DNA encoding an RNAi causing lethality or sterility. In this case, lethality may correspond to low fitness, such as flightless, rather than outright lethality, provided that the likelihood of breeding on is substantially reduced.

4. Using site-specific recombinase(s)

Specificity can also be introduced into the positive feedback system by inserting a "stuffer" fragment which inactivates it. If this "stuffer" fragment is flanked by target sites for a suitable site-specific recombinase, then it will tend to be excised in the presence of active recombinase. Any system for selective expression of active recombinase, for example, expression of the recombinase under the control of a female-specific promoter, will therefore tend to lead to selective expression of the positive feedback system, in this case in females only. If the recombinase is expressed in somatic cells only, for example by using the method described above, then the version transmitted to the next generation includes the stuffer fragment, which can again be daughters but not sons. Conversely, if the recombinase is expressed in the genome only, provision of active recombinase will lead to offspring in which the expression system is active, from parents in which it is inactive. This can be used, for example, to generate gametes containing an active dominant lethal or sterile gene system (*e.g.* female-specific or non-sex-specific) for use in an insect population control strategy.

In a preferred embodiment, the stuffer fragment encodes the recombinase. This embodiment is particularly compact. In another preferred embodiment, the stuffer fragment encodes a transcriptional repressor which tends to inactivate the positive feedback expression system – this embodiment tends to reduce the basal expression of the system in the presence of the stuffer fragment.

Conversely, the system can be specifically inactivated in certain cells, or clones of cells, by introducing target sites for a suitable site-specific recombinase at suitable positions, and then expressing or introducing the appropriate active recombinase in appropriate cells, such that one or more key functional elements of the expression system are removed or disrupted by recombination between the target sites for the recombinase.

Suitable recombinase systems include cre/lox and Flp/FRT.

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Claims:

1. An insect gene expression system, comprising at least one gene to be expressed and at least one promoter therefor, wherein a product of a gene to be expressed serves as a positive transcriptional control factor for the at least one promoter, and whereby the product, or the expression of the product, is controllable.

2. A system according to claim 1, wherein the control factor is the tTA gene product, and wherein the tetO operator is operably linked with the promoter.

3. A system according to claim 1 or 2, wherein the promoter is a minimal promoter.

4. A system according to claim 3, wherein the promoter is derived from, or is a fragment of, CMV or Hsp70.

5. A system according to any preceding claim which substantially selectively reduces fitness when activated or de-repressed.

6. A system according to claim 5, wherein the reduced fitness is a high mortality rate.

7. A system according to claim 5 or 6, wherein the selectivity is sex specificity.

8. A system according to claim 5, 6 or 7, wherein the selectivity is developmental stage specificity.

9. A system according to any preceding claim, wherein an effector gene is operably linked with at least one said promoter.

10. A system according to claim 9, wherein the effector gene is a dominant lethal gene.

11. A system according to claim 9, wherein the effector gene encodes RNAi.

12. A system according to claim 9, 10 or 11 wherein activation of a promoter to which the effector gene is operably linked leads to a selective effect *via* a transcription or translation product of DNA under the control of the promoter.

13. A system according to claim 12, wherein the selectivity is sex specificity.

14. A system according to claim 12, wherein the selectivity is developmental stage specificity.

ABSTRACT

PEST CONTROL

Promoters active in insects can be enhanced by positive feedback mechanisms and associated with repressible lethal effects.